Drought-induced mortality in Scots pine

Drought-induced mortality in Scots pine: opening the metabolic black box

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ABSTRACT

- Forests are sensitive to droughts, which increase the mortality rate of tree species. Various processes have been proposed to underlie drought-induced tree mortality, including hydraulic failure, carbon starvation, and increased susceptibility to natural enemies. To give insights into these processes, we assessed the metabolic effects of a mortality-inducing drought on seedlings of *Pinus sylvestris* (Scots Pine), a widespread and important Eurasian species.
- In testing seedlings from provenances with varying water availability, we found divergence over time in the foliar metabolic composition of droughted vs. well-watered individuals, with the former showing increased abundance of aromatic amino acids and decreases in secondary metabolism associated with defence.
- We observed no significant differences amongst provenances in these effects: seedlings from drought-prone areas showed the same foliar metabolic changes under drought as seedlings from moist environments, although morphological effects of drought varied by provenance.
- Overall, our results demonstrate how severe drought may lead to decreases in compounds derived from aromatic amino acids and compromise secondary metabolic pathways related to defences against natural enemies, thereby contributing to the risk of drought-induced mortality in *Pinus sylvestris*.

Keywords (6-10): drought, *Pinus sylvestris*, metabolomics, plant defence, seedling, provenance effects, genotype by environment, carbon starvation, hydraulic failure

Running header (short title): Drought effects on growth and metabolism of *Pinus sylvestris* seedlings

INTRODUCTION

Fluctuations in environmental conditions necessitate appropriate plant responses. Despite documentation of widespread forest dieback triggered by drought in all major forested biomes (Allen et al. 2010; Allen et al. 2015), and of elevated mortality under drought in various tree species (Mueller et al. 2005; Breshears et al. 2009; Martinez-Vilalta et al. 2010; Rigling et al. 2013), the underlying mechanisms of drought-induced mortality, species-specific vulnerability and population level resilience are still poorly known. The theoretical framework of physiological mechanisms of tree mortality currently focuses on hydraulic failure, where fluid transport breaks down, and decreasing carbon availability, where metabolic demands may be unmet owing to depletion of non-structural carbohydrates (e.g., McDowell et al. 2008; Galiano et al. 2011; Körner 2015; Adams et al. 2017). Depending on the tree species, these mechanisms need not be mutually exclusive (McDowell et al. 2008; Salmon et al. 2015; Mencuccini et al. 2015). For example, mortality of *Pinus edulis* was found to occur through both hydraulic failure and carbon starvation (Sevanto et al. 2014). Further to these hypotheses, owing to the coupling of xylem and phloem fluid transport, phloem transport limitations under drought have been suggested to contribute to failure to supply the non-structural carbohydrates (NSC) essential to plant metabolism (McDowell & Sevanto 2010; Sevanto et al. 2014). Defining carbon limitations and carbon starvation prior to death has proven hard (e.g., Sala et al. 2012; Sevanto 2014), because little is known about the multiple metabolic pathways via which energy flows to cover the metabolic needs of plants and because of the many ways by which carbon and water limitations can interact in plants.

Owing to high mortality levels during seedling establishment, this stage represents a major bottleneck to recruitment into a population. Seedling establishment has been used as an indicator of the effects of climate change on species assembly in plant communities (Sternberg et al. 1999; Kullman 2002; Lloret et al. 2009). However, despite the heightened mortality rates and increased sensitivity to climate change of this demographic, the mechanism of drought-induced mortality at the stage of seedling establishment is understudied (Lloret et al. 2004, 2009).

Scots pine is widespread across Eurasia, serving as a key timber species and abundant and ecologically important in natural forest stands. Scots pine populations are known to vary ecotypically (Rehfeldt et al. 2002). Latitudinal and longitudinal clines of phenotypic variability in physiological traits have been observed, as well as differences in adaptive plasticity across

provenances (summarised by Semerci et al. 2017). Both phenotypic plasticity and local adaptation could have a role in enabling resilience to drought. Relative to structural and morphological plasticity, metabolism changes over short time-scales and is a more immediate reflection of a plant's response to environmental stressors, including drought. Therefore, a dynamic picture of a plant's response to drought over time can be obtained in metabolic studies, both within and across provenances, which may shed light on how drought-induced mortality is related to particular metabolic pathways. Drought stress is discernible at the level of plant metabolic phenotypes. For example, shifts in carbon metabolism and secondary metabolite synthesis related to water deficit and oxidative stress have been detected in multiple plant species (Zhao et al. 2015, Bowne et al. 2012, Ings et al. 2013, Gargallo-Garriga et al. 2014). Oxidative stress caused by the accumulation of cytotoxic reactive oxygen species (ROS) may be of particular relevance to metabolic activity during mortality-inducing drought, since the extent of oxidative damage is governed by the activity of particular metabolic pathways and the capacity of antioxidant defences to avert an imbalance of ROS (Cruz de Carvalho et al. 2008).

Although the effects of drought on the metabolome of woody plants have been investigated before (e.g., Gargallo-Garriga et al. 2014, 2015; Hamanishi et al. 2015, de Simón et al. 2017), investigation of metabolic changes during drought to the point of mortality has not been carried out. Because the levels of carbon reserves may change and even increase during drought, investigation of the metabolic responses just prior to death are required to determine the sequence of metabolic events leading to mortality (Ryan 2011). Non-targeted metabolomics offers the possibility of capturing a global picture of metabolism, rather than just that related to known metabolic pathways, and it can aid the discovery of novel pathways and interactions amongst pathways (Hall 2006). Both phenotypic plasticity and local adaptation could have a role in enabling resilience to drought and the seedling response is of particular importance, since it represents a bottleneck in terms of higher mortality rates (Castro et al. 2005; Matias et al. 2011; Semerci et al. 2016). Despite increasing knowledge on plasticity and genetic variability of morphological and physiological traits, very little is known about plasticity at the metabolic level. Insights into metabolic changes during seedling drought-induced mortality will help to address this knowledge gap.

Here, we conduct an in-depth comparison of the foliar metabolome of *P. sylvestris* seedlings that were droughted to the point of death versus well-watered, control seedlings. Further, we

compared seedlings from provenances that differ in natural water availability, in order to assess if there is provenance-specific variation in metabolic responses to drought. Additional measurements of biomass and functional traits were taken to determine if changes at the whole-plant level correspond to those observed at the metabolic level. We hypothesise that drought will have major effects on plant metabolism when photosynthesis becomes compromised and carbohydrate availability for plant defence pathways is restricted. We also hypothesise that seedlings from provenances that do not regularly experience intense droughts will show greater metabolic and whole-plant changes under the drought treatment.

MATERIALS AND METHODS

Experimental conditions and sampling

The experiment was carried out over 5 weeks in July and August of 2015 in a controlled growth chamber at the University of Edinburgh (UK), under constant conditions with diurnal cycles of 16 h light at 100 μmol m^{-2} s^{-1} and 8 h darkness. The light level was chosen to reduce the rate of water use and consequent dehydration of the plants during the drought, as potted plants would otherwise rapidly undergo hydraulic failure in a manner not reflective of field conditions (Adams et al. 2017). Relative humidity was 65% (day) and 50% (night), with a constant day/night temperature of 21˚C. This is the current mean temperature at the southern range limit of this species during July and August (Matías and Jump 2014). The experiment was carried out on 10 month old seedlings, germinated from seeds originating from four provenances spanning a gradient of water availability from wet (Scotland) to intermediate (Austria, Poland) to dry (Spain). Climatic characteristics of the four populations from which seeds were collected are presented in Table 1. Mean annual precipitation was obtained from 1901 to 2015 from the CRU TS3.10 Dataset (Harris et al. 2014). Maximum climatic water deficit values for these sites, which correspond to the summed difference between evapotranspiration and precipitation over the dry season, were obtained from Chave et al. (2014). To give an indication of the degree of water stress expected at the various seed-collection sites, mean soil pF values in the summer month with the maximum mean pF value (July/August) from 1990 to 2014 were obtained from the European Drought Observatory (edo.jrc.ec.europa.eu/ on February 2nd, 2015). pF expresses the force with which different quantities of water are retained in the soil (Woodruff 1940), with lower values indicating higher water availability for plants. The seeds were collected from open-pollinated trees, with at least 5 maternal parent trees sampled per provenance site. A total

of 1200 seedlings were included in the experiment, with 600 per treatment and 300 per provenance.

Two months prior to instigating drought and control treatments, the seedlings were re-potted into 7 x 7 x 8 cm pots with Levingtons M3 pot and bedding high nutrient mix (Everris, Ipswich, UK). The drought treatment consisted of complete withdrawal of irrigation that resulted in a steep decline in soil water content (Fig. S1). A set of 10 seedlings were droughted one week before the others and were used to indicate when a final sampling point prior to mortality should be carried out. By the end of the first week after the final harvest, 80-90% of seedlings in the drought treatment had died, while mortality had not yet started by the time the final samples had been collected. A one-week, re-watering period was used to confirm that the seedlings were indeed dead. During the experiment, 40 pots across treatments and provenance (5 pots per provenance and treatment) were weighed at 09:00 on days 0, 14, 29 and 36. At the end of the experiment following plant harvesting, the pots were oven dried at 70°C for 48 hours to obtain the dry weight. The gravimetric soil water content (θ_d) (grams of water per gram of oven-dried soil) was calculated as (wet soil weight - dry soil weight) / dry soil weight. Then volumetric water content (θ_{vd}) was calculated as gravimetric soil water content (θ_d) x (bulk density (d_b) / density of water (d_w)).

Ecophysiological measurements

To assess pre-experiment morphological variation amongst provenances, height and crown depth were measured for 15 seedlings per provenance at the start of the experiment. To assess morphological impacts of drought, at each sample point including the start of the experiment (t1=0, t2=11, t3=29), 5 individuals were sampled per provenance per treatment for trait analyses. Fresh leaf weight (FW, g) was measured by separating all needles from the shoot and weighing them. After this, total leaf area (TLA, cm^2) was obtained using scanned images of all leaves and the ImageJ software (Image-J 136b; NIH, Bethesda, Maryland, USA). All needles were saturated in vials of water for 24 hours in order to obtain the turgid weight (TW, g). Dry weights (DW, g) of total needles and stem tissue were obtained after oven-drying for 48 hours at 70 °C. The percent relative water content was then calculated as: RWC = (FW-DW)/ (TW-DW) x 100. Water deficit was calculated as WD = (TW-FW)/ (TW-DW). Specific leaf area was calculated as SLA = TLA/ DW.

196 Entire root systems were washed and arranged so as to avoid overlapping lateral roots on a
197 clear plastic tray with a white background. Images taken with a digital camera were converted
198 to binary and analysed to obtain total root length using RootReader2D plugin (Clark *et*
199 *al.,*2013) in ImageJ. Roots were oven dried at 70°C for 48 hours and weighed to measure root
200 biomass (g). Specific root length (SRL, m g^{-1}), the ratio of root length to dry mass of roots, was
201 also calculated.

202 To determine photosynthetic responses to drought, chlorophyll fluorescence from needles was
203 measured by means of a Plant Efficiency Analyzer (Hansatech Instruments Ltd., Norfolk,
204 England). The ratio of variable (F_v) to maximum fluorescence (F_m) was taken, since this value
205 has been widely used for assessing plant physiological status and the state of Photosystem II
206 (PSII) (Murchie & Lawson, 2013). F_v/F_m was measured for 5 seedlings per provenance at four
207 time points (0, 12, 26 and 33 days). The middle portion of one needle per seedling was placed
208 in the centre of the leaf clip measuring area. Needles were detached and dark adapted in leaf
209 clips supplied with the analyser for 30 min at room temperature. Then the minimum
210 fluorescence (F_o), maximum fluorescence (F_m), variable fluorescence ($F_v = F_m - F_o$), and the
211 ratio F_v/F_m were recorded using a saturating intensity pulse for 0.7 s at 80% intensity level of
212 photon flux density (4620 µmol m^{-2} s^{-1}). The maximal quantum yield at PSII was calculated as
213 $F_v/F_m = (F_m - F_o)/F_m$.
214
215 *Metabolomics sampling, extraction and analysis*
216 To determine metabolic responses to drought, the foliar tissues of five randomly selected
217 individuals per provenance per treatment were sampled at midday at three time points (0, 11,
218 and 29 days). As the sampling was destructive, these were different individuals than those
219 sampled for trait measurements. Entire shoots were immediately frozen in liquid N_2 to ensure
220 no wounding response was elicited by removing tissue from the plant, and then stored
221 temporarily in frozen aluminium foil on dry ice. Samples were stored in a -80°C freezer until
222 metabolite extraction.
223
224 A monophasic extraction method was used to extract metabolites. The solvent comprised
225 acetonitrile, methanol, and HPLC-grade water (2:2:1) and was kept on wet ice to avoid
226 evaporation. Frozen needle tissue was weighed into MK-28 Precellys homogenisation tubes
227 (Stretton Scientific, Stretton, Derbyshire, UK) containing steel beads. The volume of solvent
228 to be added was adjusted according to the fresh tissue weight and 5 % extra acetonitrile and

methanol were added to compensate for estimated differences in relative water content between drought and control. Following the addition of solvent, samples were vortexed and homogenised in a Precellys-24 bead-based homogenizer (Stretton Scientific) at room temperature with two 3 minute pulses of 6800 rpm. The homogenate was transferred to a 1.5 ml microtube (Sarstedt, Nümbrecht, Germany) and half the volume of solvent was used to wash the Precellys tube to ensure that any residual homogenate was transferred. Samples were placed on ice and vortexed for 10 s before the mixture was centrifuged at 19 °C, 14000 rpm (19064 rcf, 10 min). Finally, 400 µl of supernatant were transferred to a new microtube, which was stored at -80°C prior to speed vacuum drying (Wu *et al.,*2008). After centrifugation, equal volumes of the supernatant were transferred into a new plastic tube and dried in a SpeedVac before storage at -80°C. Taking up the samples in 100 µl water / methanol 1:1 with 0.1 % formic acid could not be achieved by vortexing alone and required 20 min of sonication. A quality control (QC) sample was pooled from 10 µl each.

After centrifugation at 15000 rpm (21885 rcf) for 10 min at 4°C (Biofuge), 20 µl per sample were pipetted into a 96-well plate in a controlled randomised order, with QC samples placed equidistantly amongst them. The samples were analysed by UHPLC-MS on a Thermo Scientific Q Exactive mass spectrometer attached to a Thermo Dionex Ultimate 3000 RS system, equipped with a Thermo Hypersil Gold column (100 x 2.1 mm, 1.9 µm particles) (Clark et al. 2017). Solvent A was 0.1 % formic acid in water and Solvent B was 0.1 % formic acid in methanol. Liquid chromatography was performed over 14 min at a flow rate of 400 µl/min containing a gradient from A to B from 1 to 8 min. Following analysis of preliminary test samples of aqueous and organic phases, data were collected in positive ion and profile mode, *m/z* (mass to charge ratio) 100-1000 Da at 70,000 resolution. Three additional runs of the pooled QC sample were performed, one in positive ion mode at higher resolution (140,000), and two runs using data dependent acquisition; MS/MS fragmentation dependent on the highest 5 signals per MS scan, in positive and negative ion mode. MS data were converted into mzML format using MS Convert, and an R based XCMS/Camera script was used to obtain a first raw intensity matrix, which was imported into MatLab (SimStitch 3.1). Blank filtering was applied using a two-fold sample over blank threshold and peak signal filtering used an 80 % sample filter, applied per group to generate a Sample Filtered Matrix. The dataset was normalised using the probabilistic quotient normalisation (PQN) algorithm to correct for peak intensity differences. Missing values were imputed using a K Nearest Neighbour algorithm (k=5). All

values were then transformed using a generalised logarithm (g-log) to minimise heteroscedasticity in downstream statistical analyses (Parsons et al. 2007; Di Guida et al. 2016).

Statistical and bioinformatics analyses

In order to assess the effect of drought treatment, provenance and their interaction on ecophysiological traits, we conducted 2-way analyses of variance (ANOVAs). Initial analyses using all time points in the model and time as factor showed that most of the variation in response variables occurred at the third time point and we thus focused on contrasting results for the second and third time points. Where necessary, variables were log or arcsine transformed prior to analysis to improve the normality of model residuals. This applied to: crown percentage of shoot, maximum root length percentage of total root length, leaf RWC and F_v/F_m data.

Metabolomics data were analysed using a combination of multivariate and univariate statistics. A visual comparison of the QC samples in multivariate space generated by a principal component analysis (PCA) assured us of the technical quality of metabolomics profiles (Fig. S2). The QC samples were then removed from the data for further analyses. We next conducted an additional exploratory PCA to visualise how the overall metabolome varied among provenances, experimental treatments and time points.

To test for the overall response of the metabolome during the experiment, we used regularised multiple analyses of variance (rMANOVA). The combined implementation of multiple analytical approaches has been strongly recommended when analysing metabolomics datasets (Karp et al. 2005; Goodacre et al. 2007; Vinaixa et al. 2012). Multivariate analysis of variance (MANOVA) cannot be used for analysis of high-dimensional data where the number of observations is (much) less than the number of variables. This issue is avoided by using regularized MANOVA (rMANOVA), a multivariate data analysis method that has been specifically developed for analysis of multi-factor untargeted metabolomics data (Engel et al. 2015). rMANOVA can be considered as a MANOVA where a regularized (shrinkage) estimator of the within-group variation is used rather than the sample estimator. Because of this, the method is applicable to data where the number of observations is much smaller than the number of variables. This method is closely related to ANOVA simultaneous component analysis (ASCA), which is a well-known method for analysis of such data. The difference between rMANOVA and ASCA is that rMANOVA tries to better take the correlations between

296 the observed peak intensities into account, which often makes it a more sensitive method to
297 detect significant differences among groups (Engel *et al.,*2015). We constructed a full model
298 with factors drought, time point, and provenance, including all possible two-way and three-
299 way interactions. A permutation test with 1000 permutations was used to assess the
300 significance of each factor and interaction in the rMANOVA model (Matlab, R2014b).
301
302 ANOVAs, in combination with Benjamini–Hochberg false discovery correction at $\alpha = 0.05$
303 (Benjamini & Hochberg 1995), were used for univariate analyses of the data. As provenance
304 did not generally show any significant results on its own or in interaction with other factors,
305 we focused on the effects of sampling time point and drought treatment on metabolite
306 composition of individuals. Specifically, we conducted pairwise comparisons that contrasted
307 different time points and experimental treatments. We first determined the number of
308 metabolites that showed significant differences in abundance between groups, as measured by
309 metabolite peak height.
310
311 For metabolite identifications, we focused on metabolites that showed significant differences
312 in abundance between drought and control at time point 3 and had an average absolute $\log_2$
313 fold change value greater than 1 in this comparison (i.e. were twice as abundant or twice as
314 rare in droughted versus control seedlings). We focused on this comparison because
315 multivariate and univariate analyses indicated that the greatest differences in metabolic
316 composition were between individuals in drought versus control at time point 3 and because
317 this represents the time point at which individuals in the drought treatment were closest to
318 drought-induced mortality.
319
320 Putative metabolite annotations were carried out by matching *m/z* values to the Kyoto
321 Encyclopedia of Genes and Genomes (KEGG) (Kanehisa *et al*. 2016), LipidMaps (Fahy et al.
322 2007) and BioCyc (Caspi et al. 2016) databases. These annotations were carried out with the
323 software MI-PACK using a 2 ppm (KEGG with molecular formula search up to *m/z* 620),
324 3 ppm (BioCyc) or 4 ppm (LipidMaps) error margin, thereby also defining a range of
325 reasonable molecular formulae (Weber & Viant 2010). Since isomers and adducts are included
326 and not always distinguishable, and the number of possible compounds increases over the *m/z*
327 range, not all peaks could be assigned with putative metabolite identification. The LC-MS/MS
328 data collected assisted the annotation (level 2 identification; Sumner *et al.* 2007).
329

To aid in identification of metabolic activity of specific pathways, given incomplete knowledge of individual metabolite identities, the library *mummichog* (Li et al., 2013) from the *MetaboAnalystR* package (Xia et al., 2015) was used. *Mummichog* employs a probabilistic framework to bypass the need for complete prior metabolite identification, by combining knowledge of metabolite spectral features with knowledge of linkages among known metabolites within curated metabolic pathways (based on putative annotations). Given a list of *m/z* ratios, values of log-fold change from control and univariate tests of significance for individual compounds, *mummichog* computes a list of all possible candidate metabolites, including common isotopic derivatives and adducts. If the list of significant features reflects true metabolic activity, the true metabolites should show 'enrichment' (i.e., a significant increase in the treatment relative to the control, or vice versa) for particular pathways, while the falsely matched compounds should be distributed more randomly. In practice, *mummichog* calculates a contingency table of enriched metabolites for every known pathway relative to the total number of metabolites in that pathway; it adjusts the Fisher t-test by incorporating the EASE measure of pathway size to increase robustness and draws random permutations of all listed metabolites (including falsely matched compounds) across all pathways to derive a gamma null distribution of P values, which is tested against the observed enrichment list. To identify the metabolic pathways most likely altered immediately prior to death, we employed *mummichog* to test for enrichment in the control against the drought treatment for the last time point of the experiment. We ran *mummichog* in positive analytical mode, assuming a mass accuracy of 3ppm and running 1000 permutations for each run. We considered cut-off probability points varying from 0.05 to 0.0001 for the significance of the gamma distribution and examined which pathways were most consistently identified across all cut-off thresholds (fewer pathways are identified with lower thresholds). Software R version 3.2.2 was used for data analyses (R Core Team, 2015).

RESULTS

Seedling biomass and photochemical capacity

At the start of the experiment (day 0, time point 1), there were significant differences amongst provenances in height, with the tallest seedlings being from the Austrian provenance, Pernitz (ANOVA, d.f=3, F=3.09, p=0.03). There were marginal differences in SLA, with the highest SLA in seedlings from the Scottish provenance, Rothiemurchus (ANOVA, d.f=3, F=2.81,

p=0.072). Provenances did not differ significantly in any other seedling traits at the start of the experiment ($p > 0.1$). At time point 2 in our study (day 11), most morphological variables were not significantly affected by the drought treatment or provenance. Two exceptions were a significant difference between drought treatment and control for root to shoot ratio (ANOVA, d.f=1, F=11.08, p=0.002) and significant differences among provenances for maximum quantum yield of PSII, Fv/Fm (ANOVA, d.f=3, F=2.9, p=0.04). There were no significant interactions of treatment by provenance at time point 2.

By time point 3 (day 29), greater morphological differences between drought and control treatments were apparent. There was a significant decrease in total, shoot and root dry weight of seedlings under drought, as well as significant provenance interactions with the drought treatment (Table S1; Fig. 1A-C). Seedlings from Rothiemurchus were most affected under drought and showed reduced total, shoot and root dry weight, while the least reduction in biomass was found for the Spanish provenance. Root to shoot ratios significantly decreased under drought, though no provenance interaction effect was found (Fig. 1D). Drought treatment significantly decreased the specific root length and maximum root length by day 29 of the experiment, while no provenance effects were detected (Table S1; Fig. S3). Drought also significantly increased the maximum root length as a fraction of the total root length by day 29 of the experiment (ANOVA, d.f=1, F: 11.1, p=0.002) (Fig. S4). A significant effect of drought treatment on F_v/F_m ratios was found after day 26 (Fig. S5). There was also a provenance interaction with drought on F_v/F_m ratios on day 26. F_v/F_m decreased most in seedlings from Rothiemurchus and Pernitz and was least reduced in seedlings from Sierra Nevada (Fig. S5).

Metabolomics

Our UHPLC-MS approach generated peak height data for 4640 distinct peaks that putatively represent distinct metabolites with their adducts and isotopes, with peak height representing relative metabolite abundance. As an initial analysis of the data, PCA scores plots were constructed where the samples were coloured according to the levels of the factors of interest (e.g. drought vs control). No separation between the samples was observed except for the interaction between drought and time. The drought time point 3 samples were clearly separated from the other samples (Fig. S6). A principal component analysis-based reduction of the metabolomics data gave two main components that together explained 20% of the variation in the data, while a scree plot showed a sharp reduction in variation explained by subsequent axes.

A visual assessment of these two principal components (Fig. 2A&B) suggests that metabolite composition did not change substantially between time points 1 and 2, but that the leaf metabolome of seedlings in the drought treatment diverged substantially from seedlings in the control treatment at time point 3. For each experimental treatment at each time point, there was substantial overlap amongst provenances in metabolite composition, becoming more pronounced as the experiment progressed (Fig. 2A&B). These visual impressions were confirmed in multivariate analyses of the data by regularised MANOVA (rMANOVA; Engel et al. 2015). This showed a significant effect of drought treatment ($p < 0.001$), time ($p < 0.001$) and their interaction ($p < 0.001$) on leaf metabolic composition of seedlings, while provenance had no significant effects, either on its own ($p = 0.981$) or in interaction with time ($p = 0.787$) or experimental treatment ($p = 0.987$).

Univariate pairwise comparisons of specific time points and treatments showed that a much greater number of metabolites differed significantly in abundance between the drought and control treatments at time point 3 (1138 signals) than at time point 2 (190 signals) (Fig. 2C). Within the drought treatment, seedlings showed a substantial difference in metabolite abundances between time points 2 and 3 (892 signals). Within the control treatment, fewer metabolites changed significantly in abundance between time points 2 and 3 (125 signals) (Fig. S7). The smallest changes in metabolite abundance were observed between seedlings at the start of the experiment and seedlings at time point 2, both in the drought (14 signals) and control (29 signals) treatments (Fig. 2C; Fig S7).

For further scrutiny and identification, we selected signals that showed a significant difference in abundance under drought vs. control at time point 3, and that had a $\log_2$ fold difference in abundance greater than one (i.e., were twice as abundant or twice as rare under drought vs. control at time point 3). The selection of these metabolites is visualised in a Volcano plot (Fig. 3). Overall, more metabolites experienced a significant and substantial decline under drought ($\log_2$ fold change >1) than a significant and substantial increase (183 vs. 146 signals). Yet, among the metabolites that changed the most under drought vs. control, those that increased showed a greater absolute magnitude of change than those that decreased (Fig. 3). For example, among the top 25 signals in terms of absolute magnitude of change, 23 of those increased under drought vs. control, while only two decreased.

We were able to obtain putative annotations for a minority of the signals that showed significant decreases or increases in abundance under drought vs. control. Of the 183 peaks that were significantly less abundant under drought and show a $\log_2$ fold change less than -1 (i.e. are at least twice as rare on average in drought vs. control at time point 3), only 29 were matched in the KEGG, LIPDMAPS or BIOCYC databases. Of the 49 signals with the greatest decrease under drought relative to control, only 10 have putative annotations in these databases (Table 2; see Table S2 for full list). Of the 146 peaks that are significantly more abundant in drought vs. control and show a $\log_2$ fold change of at least 1 (i.e. are at least twice as common on average in drought vs. control), 98 have no putative annotations in the KEGG, LIPIDMAPS or BIOCYC databases. Of the 22 compounds with the greatest increase under drought relative to control in terms of fold-changes, only 10 have putative annotations (Table 3; see Table S3 for full list). The metabolites that were more abundant under drought were mainly aromatic amino acids, which may signify up-regulation of aromatic amino acid biosynthesis via the shikimate pathway or protein degradation under drought stress. A number of upregulated signals coeluting at 4.08 min are likely breakdown products of only one metabolite, tryptophan (manual interpretation).

Pathway analysis with *mummichog* identified the following pathways as the primary sites of metabolic enrichment immediately prior to death: aminoacyl-tRNA, phenylalanine (Phe), vitamin B6, phenylpropanoid, isoquinoline alkaloid, arginine and proline (Pro), Phe and Tyrosine (Tyr) and Tryptophan (Trp) biosynthesis (Table S4). Interestingly, the amino acids synthesised via the shikimate pathway (Tyr, Trp and Phe) clearly increased during drought, while several of those in the downstream pathways from shikimate decreased, although the downstream response was compound-specific. Many of the significant decreases during drought were seen for mevalonate and compounds in the mevalonate/MEP-DOXP (or non-mevalonate) pathways, such as carotenoids, sterols, ecdysones, terpenoids and glycosides with a second active group of the same type.

DISCUSSION

This study investigated the foliar metabolic responses of *Pinus sylvestris* seedlings to severe drought, which eventually led to drought-induced mortality, and to ascertain whether ecotypic adaptation in the metabolic responses to drought would be discernible amongst provenances. Pronounced metabolic effects of drought were found, potentially related to a decoupling of

carbon assimilation and secondary metabolism as photosynthesis became compromised. This manifested through an increase in free amino acids and a decrease in secondary metabolite production via the shikimate and mevalonate/MEP-DOXP pathways. Our second hypothesis was not supported, as we found no significant foliar metabolic differences amongst provenances in the response to drought. The latter is particularly interesting in light of the fact that we found morphological responses to drought to vary by provenance. Seedlings from the driest provenance were least affected morphologically by drought.

Seedling foliar metabolome response to drought

There were significant differences in the metabolite composition of the control and drought treatment groups, with a clear divergence in metabolic profiles following four weeks of drought stress after which mortality rapidly ensued (Fig. 2). Free amino acids (Tyr, Trp and Pro; Table 3) produced during primary metabolism were identified by ANOVA and rMANOVA. Tyr and Trp increased with the highest fold change under the drought treatment. Pathway analysis with *mummichog* highlighted the aminoacyl t-RNA pathway as enriched, owing to the abundance of free amino acids. However, the inference of increased t-RNA synthesis based solely on the presence of free amino acids seems untenable, especially given that drought stress conditions would presumably have impeded protein synthesis. Besides the already mentioned aromatic amino acids Tyr and Trp, significant increases were also found in several other amino acid metabolic pathways (i.e., phenylalanine, proline, glutamine, valine, leucine, isoleucine, arginine, histidine), leaving only the metabolic pathways of cysteine, methionine, glycine, serine and threonine unaffected. The shikimate pathway involved in aromatic amino acid biosynthesis was identified as a dominant pathway by *mummichog*. Plants synthesise aromatic amino acids, such as Tyr and Trp, via the metabolically costly 7-step shikimate pathway, to which over 30% of photosynthetically derived carbon can be directed (Maeda & Dudareva 2012). Secondary compounds involved in plant defence and abiotic stress responses can be derived from shikimate. It is conceivable that, rather than biosynthesis via the shikimate pathway, protein degradation accounts for the increasing levels of free amino acids detected. The higher abundance of aromatic amino acids relative to other amino acids however seems to indicate that the shikimate pathway is involved. Nevertheless, a concomitant increase in shikimate and chorismate intermediates was not found, which has been used to confirm involvement of the shikimate pathway in accumulation of free aromatic amino acids (Tyr, Trp and Phe) in drought sensitive wheat leaves (Michaletti *et al*. 2017). Pathway analysis

furthermore identified the mevalonate pathways in secondary metabolism as most affected by drought, with decreases in key metabolites involved with plant defence.

Tyrosine (Tyr) was the metabolite that showed the strongest increase under the drought treatment. Tyr hyperaccumulation in young shade leaves of *Inga umbellifera* has been linked to decreased insect larval performance, thus representing a rare example of an amino acid functioning as a defensive compound (Lokvam *et al.,*2006). Among the annotated metabolites, tryptophan (Trp) showed the third greatest increase under drought versus control. Trp biosynthetic enzymes have been shown to be up-regulated in response to oxidative stress treatment in *Arabidopsis* (Zhao *et al.,* 1998). The increase in aromatic and non-aromatic amino acids, some of which are key precursors of important defence and antioxidant pathways, occurred while several defence compounds decreased (Table S4). Significant decreases also occurred for phosphomevolanate and other downstream compounds that have similar biological roles.

For coniferous species, carbon-based secondary metabolites, terpenoids and phenolic compounds are expressed constitutively and are inducible to high concentrations and provide effective defence against many pests and pathogens (Keeling & Bohlmann 2006). Decreases of secondary metabolites were detected at 29 days of drought, by ANOVA and rMANOVA. This decrease in metabolites involved in defence during the last phase of drought may reflect a decreased capacity to employ the mevalonate pathways at a stage during drought where photosynthesis and growth were already strongly impacted, and carbohydrate availability was limited.

Increased free amino acids under stress may also be the result of protein breakdown, rather than *de novo* biosynthesis of amino acids. For example, branched chain amino acids and aromatic amino acids (Tyr and Phe) have been shown to increase under osmotic stress via protein degradation in *Arabidopsis thaliana*, with a higher fold change than other amino acids; this increase was potentially owing to lower basal levels of branched chain amino acids and aromatic amino acids prior to stress induction, or slower catabolism subsequently (Huang & Jander 2017). In *Arabidopsis*, accumulation of aromatic amino acids occurs more rapidly than branched chain amino acids (Fàbregas and Fernie 2019). It is possible that the accumulation of free aromatic amino acids has a protective role under conditions of elevated oxidative stress, by scavenging free radicals (Stadtman & Levine 2003). Indeed, 2-phenylacetamide was ranked

as the second most elevated metabolite under drought and is a product of oxidation of the aromatic amino acid phenylalanine. However, the pool size changes in amino acids may simply represent an artefact of protein breakdown coupled with differential rates of amino acid catabolism. Under conditions of carbon starvation, catabolism of amino acids would provide a source of energy. The degradation pathways (catabolism) of aromatic amino acids Phe and Trp are not well-documented in plants (Hildebrandt et al. 2015). A comprehensive study of amino acid contents that included low abundance amino acids (such as aromatic ones) showed that the amount of aromatic amino acids produced by protein degradation was sufficient for secondary metabolite production, with the exception of Trp under certain stress conditions (Hildebrant et al. 2018). Proteolysis can account for the accumulation of amino acids under osmotic stress and fast catabolism of Lys as well as branched chain amino acids has been shown to be induced to provide alternative respiratory substrate during drought stress (Araujo et al. 2011; Batista-Silva et al. 2019; Pires et al. 2016). Thus turnover of amino acids may be explained in terms of proteolysis and tightly regulated amino acid metabolism.

One of the compounds with the largest increase under drought was proline. Proline is an amino acid that functions as a compatible solute, carrying no net charge at physiological pH, by raising osmotic pressure in the cytoplasm and stabilising proteins and cellular membranes (Szabados & Savoure 2010). Additionally, proline has been indicated to exhibit antioxidant capacity in free radical scavenging (Smirnoff & Cumbes 1989). Whereas aromatic amino acid accumulation under osmotic stress was linked to protein breakdown in *Arabidopsis*, increased biosynthesis accounted for elevated levels of proline (Huang & Jander 2017). Indeed, proline is biosynthesised in a number of plant species under drought and has been shown to exhibit osmoprotective and antioxidant capacities (Hayat *et al.,*2012). Furthermore, it is suggested that proline accumulation under stress may have a role in signalling as well as maintenance of NAD(P)/NAD(P)H ratios that enable metabolic pathways to function in generating secondary metabolites (Hare & Cress 1997). Biosynthesis of proline under water deficit occurs in an abscisic acid-dependent manner and can also be influenced by sugar availability, indicating a regulatory mechanism to restrict proline accumulation when carbohydrate status is low (Rook *et al.,*2001, Verslues & Bray 2006).

A number of important, significant peaks determined by univariate and multivariate statistics were not identifiable through interrogation of metabolic databases, possibly owing to taxon specificity and the poor development of metabolite databases for non-model plant species. This

563 is in addition to adducts and breakdown products often not covered by common databases. This
564 finding highlights the difficulty of current metabolomics approaches, at least for conifers, and
565 suggests that insufficient understanding of the metabolic pathways affected by drought is
566 currently hindering our understanding of the nature of carbon limitations prior to drought-
567 induced mortality.
568

569 *Provenance variation in the seedling drought response*
570 Total seedling biomass was reduced under drought treatment and this reduction varied
571 significantly across provenances. Seedlings from the wettest provenance, Rothiemurchus
572 (Scotland), were most affected under drought in terms of biomass reduction, while seedlings
573 from the driest provenance, Sierra Nevada (Spain), were least affected. Under drought, total
574 leaf area was significantly reduced, but there was no observed needle abscission. Rather, the
575 drought is thought to have inhibited needle growth. Needles also showed a reduction in the
576 maximal efficiency of photosystem II (F_v/F_m) by day 26 of the drought treatment, indicating
577 that photosynthesis was compromised, with the wettest provenance being most affected. F_v/F_m
578 declines when water stress becomes severe (Epron & Dryer 1992; Iijima *et al.,*2006; Ditmarova
579 *et al.,*2010; Way *et al.,*2013). Potentially this could be a result of decoupling between
580 photosynthesis and secondary metabolism, with C limitation once assimilation is not occurring
581 owing to stomatal closure in this isohydric species.
582

583 A limitation of this study is the lack of information on how mortality rate varies by provenance.
584 Matias and Jump (2014) found that mortality rate of *Pinus sylvestris* under drought is strongly
585 affected by temperature, with the southern Spanish provenance showing higher survival
586 probabilities than the northern Finnish provenance under drought at the current temperature
587 regime of the southern range limit. This effect may not be significant in our experiment owing
588 to the shorter timeframe (5 weeks compared with 19 weeks), since Matias and Jump found no
589 significant effect of temperature on provenances until after 5 weeks of treatment. Survival is
590 vital in determining seedling recruitment at the population level, thus if mortality rate is
591 relatively constant among provenances over short intense drought episodes, then the superior
592 drought response of the Spanish provenance in terms of morphological and physiological traits
593 would not have adaptive significance.
594

In contrast, metabolic profiles were not found to differ significantly by provenance, a result robust to changes in the signal filter threshold employed. Previous studies on intraspecific variation in conifers have found evidence for a stronger environmental than genetic signal on metabolomes of developing xylem in *Pseudotsuga menziesii* (Robinson et al. 2007). However, differences in foliar metabolomes of *Pinus pinaster* were found to be strongly related to the aridity of the provenance site of origin, suggesting local adaptation (Meijon et al. 2016). Also, Du et al. (2015) found that *Pseudotsuga menziesii* seedlings showed a provenance specific drought response, with the drier provenance increasing aromatic amino acids. In this study, the metabolic phenotypes of *P. sylvestris* seedlings do not appear to be population specific or to show local adaptation, or at least such differences did not have a sufficiently large effect size to be statistically detectable. *Pinus sylvestris* is a widespread species with high plasticity evident across European populations in traits related to physiology, phenology and morphology (Olekysn et al. 1998, 2000; Semerci et al. 2017), but the findings of this study suggest a limitation of foliar metabolic plasticity to react to extreme mortality-inducing drought episodes. The relatively long mean leaf life span of *Pinus* species (over 3 years) may account for the discrepancy between the drought response in foliar metabolic profiles and other measured leaf traits. In evolutionary terms, leaf longevity has a pivotal role in the leaf economic spectrum, with trade-offs between persistence and productivity constraining both morphological and biochemical leaf traits (Warren and Adams 2000; Onoda et al. 2017). Plasticity in needle longevity is known for *Pinus sylvestris* (Pensa and Jalkanen 2005). Juvenile needles in three Mediterranean pine species were found to exhibit a leaf strategy to maximise carbon gain and the transition to adult needles reflected changes in traits according to the drought stress tolerance of each species (Kuusk et al. 2017). Foliar integration of traits has been found to be a function of ontogeny; canalisation of leaf traits are found in plants of reproductive age, whereas in juvenile plants weaker correlations among functional leaf traits indicate lower foliar integration and higher phenotypic plasticity (Damian et al. 2016). However, the selection of leaf traits in this study did not include biochemical or metabolic functional traits. Metabolically divergent drought responses in mature trees may be more likely than for seedlings with the same leaf age, which all show a propensity to maximise carbon gain to the detriment of environmental stress tolerance; thus, canalisation of leaf traits may have affected metabolic profiles.

Conclusions

There was a strong impact of drought at the metabolic level in *P. sylvestris* needles, with the effect becoming very apparent just before seedling mortality. In response to drought, we found increases in many free amino acids and decreased concentrations of secondary metabolites in the mevalonate/MEP-DOXP pathways. The identification of decreases in compounds derived from aromatic amino acids and secondary metabolic pathways related to plant defence shows that these metabolically costly pathways are down-regulated under drought stress, revealing the incapacity of severely droughted seedlings to produce defensive compounds against biotic stress. However, proline, a compound important for osmoregulation, plant signalling and antioxidant defence, was strongly upregulated, suggesting that its high concentration was important in the latest phases of survival prior to death. While seedling biomass and photochemical efficiency were found to be most strongly reduced by drought for Rothiemurchus, the wettest provenance, there was a lack of provenance effects on metabolite abundances. Overall, our findings indicate that important metabolite changes under drought were centred around the shikimate and the mevalonate/MEP-DOXP pathways. It also demonstrates that a large number of unknown compounds were affected by drought and are therefore of interest for future research.

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Author Contribution

S.M and K.G.D. designed and executed the experiment; S.M measured plant traits and extracted the metabolites; U.S and J.E led the metabolomics analyses; K.G.D and M.M conducted additional analyses; S.M wrote the manuscript; and all authors contributed to revisions.

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FIGURE LEGENDS

Figure 1 Total biomass (A), shoot dry weight (B), root dry weight (C), and root to shoot dry weight ratio (D) on day 29 of the experiment (n = 10 for all groups). Provenances are: Rothiemurchus, Scotland (RM), Pernitz, Austria (PER), Jarocin, Poland (JAR), and Sierra Nevada, Spain (SN). Boxplots represent the median of the data and the lower and upper quartiles (25% and 75%). Whiskers represent the most extreme data point that is no more than 1.5 times the interquartile range from the box. Control treatment is grey while drought treatment is black. Different letters indicate significant differences ($p<0.05$).

Figure 2 Figure 2A) Mean ± standard errors for principle component 1 and **B)** principle component 2 for different provenances at different time points in the drought experiment (t1=0, t2=11 and t3=29 days). These two components together explain 20% of the variation in the metabolomics data. Drought treatment is indicated by the dashed lines. Provenances are colour coded as follows: blue – Rothiemurchus; orange – Pernitz; green – Jarocin; red – Sierra Nevada. **C)** Schematic showing the number of metabolites (out of 4640) that differ significantly in abundance between given time points and treatments. The thickness of the arrows between ellipses reflects the level of the differences in metabolic composition between treatments and time points.

Figure 3 Volcano plot of the metabolite changes at time point 3 (day 29) after imposing a severe drought. Metabolites are plotted as a function of the $\log_2$(fold change) from control and as $-\log_{10}$(P value) of the change from control. Thus, positive values for fold change indicate increased abundance under drought and negative values indicated decreased abundance. Metabolites that showed a log2(fold change) smaller than +- 1 and a q-value smaller than 0.05 were selected for further scrutinity and

identification (colored in red). Non-selected metabolites are coloured grey. Note that the volcano plot displays the raw, uncorrected, p-values. The horizontal dashed line shows the FDR-corrected critical p-value ($q < 0,05$), i.e. all points with p-values smaller than the critical value (all points above the line) are the features for which the null hypothesis of no difference was rejected. The plot is partially annotated for the compounds showing the most pronounced and most significant changes immediately prior to the drought-induced mortality. Legend: Trp, tryptophan; Tyr, tyrosine; Umb, umbelliferone; Gluc, dihydrozeatin riboside glucoside; Pro, proline; Phe, phenylalanine; Skat, skatole; Ser, serine; Jasm, jasmonate; Leu, leucine; Leu-Gly-Pro, leucine, glycine, proline; Gib, gibberelline; Styr, styrene; Pyr, pyrole; Spong, spongipregnoloside; Orien, iso-orientin; Ecdys, dehydroecdysone; Thap, thapsigorgin; Osc, oscillatoxin; Spin, gentibioside spinosin; Cort, hydrocortisone caproate; Purp, purpurescin; Vit D, vitamine D; Acr, Acronycine.